Overexpression of the Chemokine Receptor CCR7 in Patients with Hepatocellular Carcinoma: Correlation with Disseminated Circulating Tumor Cells

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ABSTRACT

Disseminated circulating tumor cells (CTCs) are cancer cells that have detached from the primary tumor and survived in the circulation, thus enable the spread of cancer from its site of origin. The chemokine receptor 7 (CCR7) has been linked to tumor dissemination and poor prognosis in solid tumors. However, its relationships with CTCs in liver cancer still not clear. This study aimed to identify the relationship between CCR7 and CTCs in hepatocellular carcinoma (HCC) patients, and to assess their predictive values as noninvasive markers. Seventy-one HCC patients and 20 normal individuals were included. CTCs were detected in the peripheral blood by flow cytometry defined as CD45−CK19+CD90+ cells. Expression of CCR7 was assessed by real time PCR. Clinical and routine laboratory investigations included tumor size and number of tumors detected by ultrasound. Also, alpha fetoprotein (AFP), CBC, PT, INR, ALT, AST, bilirubin, albumin, and creatinine were analyzed. Results indicated that HCC patients were classified according to their Childs-Pugh score system (CPSS) into 2 subgroups A5 (N=51) and A6 (n=20). It was found that CCR7 mRNA increased significantly in HCC patients and its elevation was correlated with CTCs count. Besides, there were significant differences in CCR7 mRNA and in CTCs between the studied groups. Both CCR7 and CTCs were significantly correlated with age, levels of ALT, and AST and negatively with platelets and serum albumin. CCR7 mRNA was correlated significantly with total bilirubin and tumor size, while CTCs was significantly correlated with AFP and INR. No significant difference between both groups regarding kidney function tests. The diagnostic efficiency of CTCs and CCR7 was assessed using ROC curve, where it was clear that CCR7 at cut off >1.02 could discriminate between patients and control with 93.1% sensitivity, 78.8% specificity, 88.5% PPV and 86.7% NPV, while CTCs concentration> 3.5 is the cutoff between patient and control groups with 91.4% sensitivity, 81.8% specificity, 89.8% PPV and 84.4 % NPV. The current results indicated that each of CCR7 and CTCs can be used as an efficient diagnostic marker, and both complement in reflecting different liver reserve status. Conclusion: CCR7 expression is increased with HCC progression. The combined assessment of CTCs and CCR7 could be considered as potential noninvasive biomarkers for HCC progression. Additional research with a greater number of patients is needed on this topic.

Keywords: Hepatocellular carcinoma, circulating tumor cells, Chemokine receptor seven, polymerase chain reactions, flow cytometry.

INTRODUCTION

HCC is the sixth most common neoplasm and represents a major health challenge with an annual global death > 600,000 which makes it the third most lethal cause worldwide (Huh et al., 2002). In Egypt, studies revealed that about ninety
percent of HCC cases are HCV-related (El-Kassas and Elbadry, 2022).

The lethality of HCC is linked increasingly with early metastasis, which is emerging from dissemination of tumor cells (DTCs) from the original tumor or metastatic foci that are flowing freely in the blood circulation. These cells are considered the drivers of recurrence and metastasis following liver cancer surgery for primary HCC (Ahn et al., 2021). These cells are often detectable in the peripheral blood as circulating tumor cells (CTCs). CTCs can lead to a new fatal metastasis and can be vividly described as “seeds” of tumors. CTCs-positive rate was directly correlated with tumor size and counts as a biomarker of poor prognosis. The absolute numbers of CTCs detected have been associated with survival and treatment response and associated with increased recurrence risk after resection and shorter overall survival as the more advanced the cancer stage, the higher number of these cells in the peripheral blood (Ou et al., 2018).

One of inflammation-related cancer is hepatocellular carcinoma, these chronic infections with hepatitis viruses (HBV and HCV) and the sustained inflammatory reactions related to the infections represent major risk factors for HCC development (Han et al., 2015). However, the chronic inflammation is characterized by the continued expression of chemokines that enables the recruitment of immune cells to the liver. When inflammatory cells are activated, they release free radicals, such as reactive oxygen species (ROS) and nitric oxide (NO) reactive species, which may cause DNA damage and cause gene mutations, thus promoting neoplastic transformation (Ma et al., 2015).

Chemokines play a vital role in tumor progression, in several metastatic tumors including HCC and colorectal liver metastasis (Jiao et al., 2019). Currently, chemokines and their receptors such as the CCR7 axis have received much research interest because it has been linked to poor prognosis and tumor dissemination because of induction of the process of epithelial-mesenchymal transition in gastric as well as ovarian cancer (Cheng et al., 2014). In addition, the CCL21-CCR7 axis was reported as an important regulator of growth and progression of esophageal cancer, as there is a strong association between the levels of their expression and the degrees of differentiation (Goto et al., 2019). Although many studies indicated that CCR7 are associated with dissemination of HCC cells (Schimanski et al., 2006), however, its relationships with CTCs and progression of HCC remains not clear. Therefore, in the present study, we aimed to assess the contribution of CTCs and CCR7 as noninvasive biomarkers for patients with HCC with distinct stages of the diseases.

PATIENTS AND METHODS
Study design and population:
The study sample includes 71 Egyptian HCC patients diagnosed by liver biopsy, CT scan, or MRI and twenty (age and gender matched) healthy subjects (control group). All subjects were recruited from the Outpatient Clinics and Inpatient of internal medicine Department of Ain-Shams University Hospitals. The laboratory investigations enumeration of CTCs and mRNA expression CCR7 were performed at the Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Egypt. All patients were subjected to through medical history and clinical examination. Laboratory investigations were performed for all participants.

Sample collection and preparation:
Blood samples were collected by venipuncture in heparin BD Vacutainer tubes and were divided into two halves, one for routine lab work and the other part for separation of the Peripheral Blood Mononuclear cells “PBMCs” that were used in RT-PCR and Flow Cytometry analyses. Buffy coats from peripheral blood samples
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were separated carefully using Ficoll-Paque plus density (1077 g/L) using “Amersham biosciences Kit” according to the manufacturer’s instructions.

Routine laboratory investigations

Complete blood count (CBC) was performed by 5-part differential automated cell counter Beckman Coulter® LH 750 (Coulter Corporation, Florida, USA), prothrombin time (PT, INR) was done on fully automated blood coagulation analyzer STA Compact Max-Stago (Asnieres Sur Seine Cedex, France). Serum ALT, AST, total bilirubin, direct bilirubin, albumin, Alpha fetoprotein (AFP), and serum creatinine were confirmed on Beckman coulter AU 480 system (Beckman coulter, Inc. 250s. Kraemer Blvd. Brea, CA92821, USA).

Estimation of chemokine receptor 7 (CCR7) by real time PCR:

RNA was extracted using the "Pure link RNA mini kit" supplied by “Ambion” (Life technologies, Carlsbad, USA) following the manufacturer’s procedure. The extracted RNA samples were eluted aliquoted into sterile tubes and stored at -80ºC until further processing. The extracted RNA was then converted to cDNA using the “High-capacity cDNA reverse transcription kit” from “Applied Biosystems” (Life technologies, Carlsbad, USA) according to the manufacturer’s instructions. Quantitative real-time PCR was done using a Light Cycler System (DT prime thermal cycler) (DNA Technology, Moscow, Russia) with CCR7-specific primers (forward):
5′-CATGTCCTACCTTCTTTGCATC-3′; and (reverse) 5′-CCTGTGCTAGTATCCAGATG-3′ Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward):
5′-ACCCAGAAGACTGTGGATGG-3′; (Reverse) : 5′-TCTGACGGCAGTCAAGT-3′ was amplified as an internal control “Ambion” (Life technologies, Carlsbad, USA). The PCR mix contained 3.2 μL Nuclease free H2O, 0.8 dNTP mix, 2 μL RT random primer, 1μl multiscribe™ reverse transcriptase, 2 μl RT buffer and 1μL RNase inhibitor. Samples with the master mix were placed into the rotor of the Light Cycler for amplification. PCR cycles were performed using the “Maxima SYBR Green q PCR master mix” supplied by “Thermo Fisher Scientific” (Life technologies, Carlsbad, USA). CCR7 mRNA concentration was calculated using the delta-delta Ct method, also known as the 2–ΔΔct method, in which the following equation was used: CCR7 RNA concentration = 2Δct (Δ ct = CCR7 reading – GAPDH reading) to calculate the relative fold CCR7 gene expression of samples (Livak and Schmittgen, 2001).

Identification of CTCs by Flow Cytometry

Flow cytometric measurement of CTCs was performed using “BD Accuri C6 plus” flow cytometry (BD Life sciences Inc, USA). Anti-human anti-CD45, anti-human CD90 and anti-CK19 monoclonal antibodies were used to identify CTCs as cells negative for CD45 and positive for CK19 and CD90 (CD45−CK19+CD90+) in the separated mononuclear layer. The fluorescence of the circulating tumor cells is analyzed to differentiate between the positively stained cells from the negative unstained ones using direct flow cytometry staining method as previously reported (Elshal et al., 2016). The results were then expressed as numbers of CD45−CK19+CD90+ CTCs in relation to all cells acquired by the cytometer.

Statistical analysis

Results were detected using SPSS version 24. Quantitative data were examined as mean ± standard error of means (SEM). Qualitative data were expressed as frequency and percentage. Chi-square test was used to compare qualitative variables. ANOVA test was used
to compare more than two groups with Bonferroni post hoc analysis for variance between pair groups. The relationship between variables in the same group was evaluated using Spearman’s correlation coefficient test. Receiver-operating characteristic (ROC) curve analysis was used to examine the value of CTCs and CCR7 for discrimination between cases and controls. A p value ≤ 0.05 was considered statistically significant.

RESULTS

Clinical characteristics:

Ninety-one subjects that enrolled in the study were categorized into 3 groups: (patient group) 71 HCC patients [6 (8.5%) males and 65 (91.5%) females]. The patients’ ages ranged from 50-61 years old with mean of 56.20 ± 5.53 years. Twenty age and gender-matched healthy subjects were recruited as control group, [0 (0%) male and 20 (100%) female]. Their ages ranging from 31-42 years with mean of 37.00 ± 5.22 years. HCC patients were grouped according to their Childs-Pugh class into 2 groups A5 (N=51) and A6 (n=20). The clinical features and laboratory findings of the studied groups are shown in Table (1). A comparative study by ANOVA test showed statistically significant difference in all studied parameters except for total bilirubin, creatinine, and total lymphocytes count.

Real time PCR of CCR7 mRNA:

CCR7 expression was found significantly higher in the HCC-A5 and HCC-A6 patients groups compared to healthy controls (P < 0.002) (Table 1).

Table 1: Clinical characteristics and laboratory findings of the studied groups.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy</th>
<th>Child-Pugh A5</th>
<th>Child-Pugh A6</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Age</td>
<td>47.5</td>
<td>5.25</td>
<td>55.47</td>
<td>4.91</td>
</tr>
<tr>
<td>ALT U/L</td>
<td>28.05</td>
<td>1.773</td>
<td>39.24</td>
<td>3.015</td>
</tr>
<tr>
<td>AST U/L</td>
<td>27.10</td>
<td>1.676</td>
<td>44.72</td>
<td>3.306</td>
</tr>
<tr>
<td>T. Bilirubin (mg/dL)</td>
<td>1.05</td>
<td>0.050</td>
<td>1.16</td>
<td>0.059</td>
</tr>
<tr>
<td>D. Bilirubin (mg/dL)</td>
<td>0.25</td>
<td>0.099</td>
<td>0.36</td>
<td>0.064</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.15</td>
<td>0.082</td>
<td>3.50</td>
<td>0.086</td>
</tr>
<tr>
<td>INR</td>
<td>1.00</td>
<td>0.000</td>
<td>1.12</td>
<td>0.043</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.00</td>
<td>0.000</td>
<td>1.00</td>
<td>0.000</td>
</tr>
<tr>
<td>Hb (Hb) (g/dl)</td>
<td>13.10</td>
<td>0.161</td>
<td>12.45</td>
<td>0.181</td>
</tr>
<tr>
<td>T. lymph. (x 10^3/µl)</td>
<td>6.00</td>
<td>0.423</td>
<td>5.57</td>
<td>0.219</td>
</tr>
<tr>
<td>Platelets (x 10^3/µl)</td>
<td>273.95</td>
<td>10.910</td>
<td>191.02</td>
<td>10.27</td>
</tr>
<tr>
<td>AFP (ng/ml)</td>
<td>1.75</td>
<td>0.33</td>
<td>57.25</td>
<td>8.53</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>-</td>
<td>-</td>
<td>2.76</td>
<td>0.176</td>
</tr>
<tr>
<td>CCR7 (folds)</td>
<td>0.25</td>
<td>0.099</td>
<td>32.33</td>
<td>5.572</td>
</tr>
<tr>
<td>CTCs (count/ml)</td>
<td>-</td>
<td>-</td>
<td>8.79</td>
<td>0.713</td>
</tr>
</tbody>
</table>

Values expressed as mean ± standard error of means (SEM) (n=20) for healthy control and (n=51 and 20) for A5 and A6 class HCC patients. Level of significance at p<0.05.A comparative study by ANOVA with Bonferroni post hoc test. a: significant (P<0.05) compared with healthy controls, b: significant (P<0.05) compared with HCC patients with Child Pugh class A5.

CTCs enumeration by flow cytometry:

CTCs enumeration was done using flow cytometry as CD45−CK19+ cells (Fig. 1), and it was found significantly higher in A6 HCC patients compared to A5 HCC and the controls (P ≤ 0.0001).
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Correlations between biochemical parameters:
There was a significant positive correlation between mRNA expression of CCR7 and the numbers of CTCs (p<0.05) (Fig. 2). CCR7 expression levels also positively correlated with tumor size and liver functions tests ALT, AST, and T. bilirubin. Moreover, CCR7 mRNA expression was found negatively correlated with serum albumin and blood platelets (Table 2). On the other hand, there were significant positive correlations between CTCs and age, ALT, INR, and AFP. While there were significant negative correlations between CTCs and Alb and Platelet, with r values of -0.311 and -0.334 respectively (p<0.05) (Table 2).

Fig. (3): Correlation between the CTCs and CCR7 expression levels.
Table 2: Correlation between the CCR7 and different variables in case group

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CCR7 r</th>
<th>Sig.</th>
<th>CTCs r</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.277*</td>
<td>0.012</td>
<td>0.616**</td>
<td>0</td>
</tr>
<tr>
<td>ALT U/L</td>
<td>0.331**</td>
<td>0.002</td>
<td>0.269*</td>
<td>0.01</td>
</tr>
<tr>
<td>AST U/L</td>
<td>0.280**</td>
<td>0.01</td>
<td>0.219*</td>
<td>0.043</td>
</tr>
<tr>
<td>Total Bili. (mg/dL)</td>
<td>0.244*</td>
<td>0.025</td>
<td>0.114</td>
<td>0.283</td>
</tr>
<tr>
<td>Direct Bilirubin (mg/dL)</td>
<td>0.18</td>
<td>0.101</td>
<td>0.077</td>
<td>0.473</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>-0.310**</td>
<td>0.004</td>
<td>-0.311**</td>
<td>0.003</td>
</tr>
<tr>
<td>INR</td>
<td>0.163</td>
<td>0.138</td>
<td>0.209*</td>
<td>0.048</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>-0.072</td>
<td>0.514</td>
<td>0.094</td>
<td>0.379</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>-0.17</td>
<td>0.123</td>
<td>-0.054</td>
<td>0.614</td>
</tr>
<tr>
<td>T. Lymph (x 10^3/µl)</td>
<td>-0.19</td>
<td>0.084</td>
<td>-0.06</td>
<td>0.575</td>
</tr>
<tr>
<td>Platelets (x 10^3/µl)</td>
<td>-0.249*</td>
<td>0.022</td>
<td>-0.334**</td>
<td>0.001</td>
</tr>
<tr>
<td>AFP ng/ml</td>
<td>0.09</td>
<td>0.422</td>
<td>0.309**</td>
<td>0.004</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>0.412**</td>
<td>0.001</td>
<td>0.053</td>
<td>0.662</td>
</tr>
<tr>
<td>CTCs count/ml</td>
<td>0.258*</td>
<td>0.019</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Diagnostic performance of CCR7 and CTC

Using ROC curve, it was shown that CCR7 at the cut-off > 1.02 can be used to discriminate between patients and controls with 93.1% sensitivity, 78.8% specificity, 88.5% PPV and 86.7% NPV, while CTC concentration > 3.5 was the cut-off between patients and controls with 91.4% sensitivity, 81.8% specificity, 89.8% PPV and 84.4% NPV (Fig. 3).

![ROC Curve](image)

Fig. 3: Receiver operating characteristic (ROC) curve between patient and control group as regards CCR7 and CTCs.
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DISCUSSION

Chemokines, as small chemotactic cytokines, participate in much physiological function on the cell expressing their receptor; they play a vital role in the progression of HCC, as CCR7 was demonstrated mediates epithelial-mesenchymal transition and suppressing apoptosis through AKT pathway (Xu et al., 2017). Additionally, the CCL21-CCR7 axis have received much attention in HCC research stemmed from the previous work by Schimanski et al., (Schimanski et al., 2006) who reported that these circulating tumor cells are disseminated from the primary tumor in CCR7-positive HCC patients. Furthermore, Chen et al., (Shen et al., 2022) demonstrated that CCL21-CCR7 axis is a potential therapeutic target for blocking the progression of HCC.

Several HCC staging systems have been proposed for the assessment of patient’s prognosis and response to treatment (Liu et al., 2016), however survival in HCC patients was found not only dependent on stages of tumor, but also the liver functional reserve (Kinoshita et al., 2012). One of the scores that rely mainly on estimating the liver functional reserve is Child–Pugh scoring system (CPSS) (Wang et al., 2018). Interestingly, it was reported that the survival rates are different in each CPSS grade and associated with treatment response for early stages of HCC (Roberts et al., 2018). Indeed, CPSS system that has proved to be adequate for the stratification of staging systems given that the degree of liver dysfunction is one of the most important prognostic factors for HCC (Kemp et al., 2005). Additionally, Hung et. Al., (Hung et al., 2014) demonstrated that HCC group of patients with A5 score had a better overall survival rate than those with A6 group as regard the early tumor stage and higher rate of effective curative treatments.

Therefore, in the present study we investigate whether the expression of CCR7 is associated with disseminated tumor cells measured as CTCs and with other prognostic markers in HCC with Grades A5 and A6 CPSS. In our study, fifty-one patients were CPSS class A5, whereas 21 patients were class A6. After adjustment for the clinical influence factors of gender and age, we found significantly higher expression of CCR7 associated with the significant increase CTCs compared with healthy controls and through the progression of liver cancer from A5 to A6. Although there were no significant differences in CCR7 expression of CPSS class A5/A6, however CTCs and serum AFP showed significant differences between A5/A6 grades. This result goes in accordance with the studies conducted by Qin et al. (Qin et al., 2021) who established a study included many patients suffering from HCC in which elevated levels of CCR7 was found as a prognostic factor to distinguish them from those with no detected focal lesion.

The finding that of CCR7 expression is upregulated as early as CPSS grade A, on contrary to CTCs and AFP, suggests that it can be an early event for liver tumorigenesis. This suggestion is supported by the research of Zhou et al., (Zhou et al., 2022) who noticed that serum levels of CCR7 is considered a less reliable marker of HCC as its elevated levels may be related to other GIT tumors as colon cancer. Moreover, it was emphasized that CCR7 is not a specific biomarker for liver tumors as its elevations were also reported in other cancers as colorectal cancers and colorectal liver metastases (Wu et al., 2022).

Disseminated CTCs, the cells that derive from the primary or metastatic lesions and migrate into circulation and, are regarded as the “seeds” of tumor metastasis that are increased in patients with HCC with advanced stages (Schimanski et al., 2006). We observed a statistically significant difference regarding CTCs detected by flow cytometry between patients with HCC
group A5 as compared to group A6 (p<0.05), and also regarding detection of focal lesion by ultrasound. This agrees with Qi et al., (Qi et al., 2018) who reported that elevated level of CTCs and thus positive focal lesions in liver are detected in most of HCC patient. Our data also revealed the presence of positive correlation of CTCs with AST, ALT, AFP, and INR, this comes in accordance with Qi et al., (Qi et al., 2018) who additionally added that the increase in serum CTCs concentrations as in HCC is associated with affection of liver functions so it could be used for differentiation between patients with HCC and healthy individuals. Meanwhile, our results confirmed the presence of negative correlation between albumin level and platelet count and CTCs where albumin levels were low and platelet count which agrees with Haruki et al., (Haruki et al., 2018) who proved that serum level of albumin as well platelet count is decreased in patients suffering of HCC with elevated level of CTCs. This supports the previously published concept that the enumeration of CTCs with exclusive and specific biomarkers may provide better diagnostic and individualized treatment options for patients with HCC (Chen et al., 2020).

CCR7 mRNA levels were also significantly increased in HCC patients, and they were correlated with CTCs numbers and tumor size and grade. To examine the diagnostic efficiency of CTCs and CCR7, ROC curve was done between CCR7 and CTCs in discriminate between patient and control groups which revealed that CCR7 at cut off >1.02 could discriminate between patients and control with 93.1% sensitivity, 78.8% specificity, 88.5% PPV and 86.7% NPV, while CTCs concentration> 3.5 is the cutoff between patient and control groups with 91.4% sensitivity, 81.8% specificity, 89.8% PPV and 84.4 % NPV. These data indicated that each of CCR7 and CTCs is efficient diagnostic marker, and both complement in reflecting different liver reserve status. However, the pronounced role of CTCs with the expression of CCR7 in liver cancer has not been documented in the early stage (CPSS A5/A6) of HCC to the best of our knowledge.

Conclusion

CCR7 expression is increased with HCC progression. The combined assessment of CTCs and CCR7 could be considered as potential noninvasive biomarkers for HCC progression. Additional research with a greater number of patients is needed on this topic.

Acknowledgments

We would like to thank the Molecular Biology Department, Genetic Engineering and Biotechnology Institute, University of Sadat City, Egypt for providing some of the Flow cytometry facility required during this study.

REFERENCES


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undergoing EMT provide a metric for diagnosis and prognosis of patients with hepatocellular carcinoma CTCs in patients with HCC. Cancer Res., 78(16): 4731-4744.


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In the study, it was observed that the expression of Chemokine CCR7 in patients with hepatocellular carcinoma was correlated with disseminated circulating tumor cells.

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